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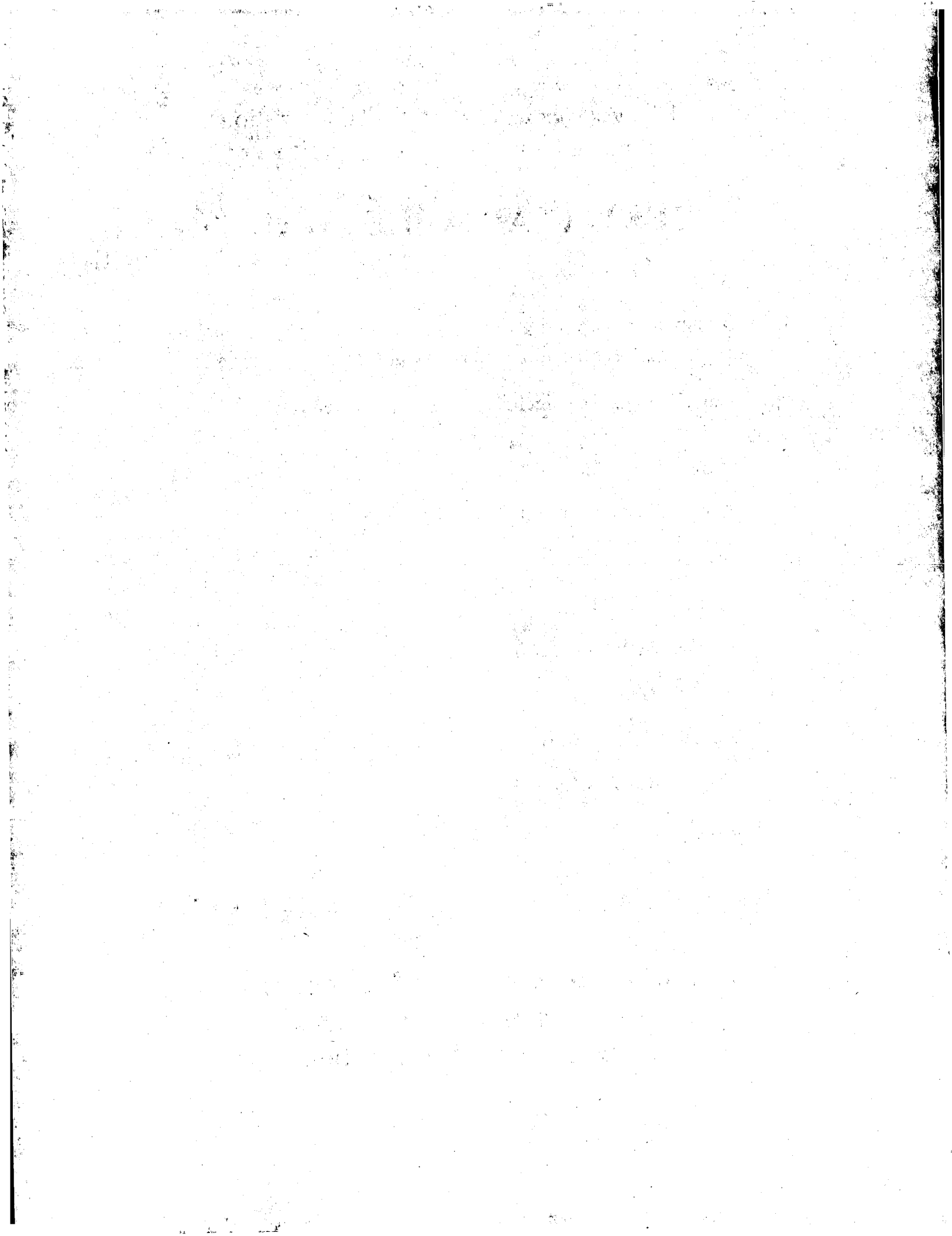
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A Hepatitis B Virus Mutant With a New Hepatocyte Nuclear Factor 1 Binding Site Emerging in Transplant-Transmitted Fulminant Hepatitis B

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Hepatitis B virus (HBV) DNA was cloned from serum of a heart transplant recipient who died from fulminant hepatitis B transmitted by the donor. Restriction enzyme analyses of the clones obtained by conventional cloning yielded six HBV variants: a major species (pF-1) representing 88% and five minor species (pF-2 to pF-6), each representing 2% to 4% of the clones. The complete nucleotide sequence of these six variants revealed that five of the six viral genomes, including pF-1, carried a novel 11 base pair (bp) insertion in the core promoter region as well as an 18 bp and an 108 bp in-frame deletion in the pre-S1 region not present in the donor. One genome was identical to the sequence of the donor. Functional analyses of HBV clones generated by *in vitro* mutagenesis and cassette exchange showed that the 11 bp insertion is a strong binding site for hepatocyte nuclear factor 1 (HNF-1). In transient transfection experiments, the novel HNF-1 sequence motif was shown to result in enhanced viral replication. Immunohistochemical analyses revealed high levels of cytoplasmic and nuclear hepatitis B core antigen (HBcAg) and only scattered hepatitis B surface antigen (HBsAg) expression in the liver. The data in our immunosuppressed patient showed that HBV variants can rapidly accumulate in severe hepatitis B and suggest that the novel HNF-1 binding site may have contributed to the fulminant clinical course, possibly via enhanced viral replication. (HEPATOLOGY 1997; 25:1507-1515.)

Organ-transmitted hepatitis B virus (HBV) infection is a major problem in transplantation surgery. The disease may take a rapidly progressive course leading to fulminant hepatic failure and death in many patients. The pathogenesis of severe liver damage in immunosuppressed patients with posttransplantation HBV infection is poorly understood. While in immunocompetent patients liver cell injury from

chronic HBV infection is thought to be immune-mediated, a direct cytopathic role for HBV is postulated in immunosuppressed transplantation recipients, especially in patients with histological features of fibrosing cholestatic hepatitis¹ and steatoviral or fibroviral hepatitis B.² In these patients high levels of viral nucleic acids and accumulation in the cytoplasm of hepatocytes of the large hepatitis B surface antigen (HBsAg) have been observed.³ Various mutations in the HBV genome have been implicated in the severe clinical course of subfulminant or fulminant HBV infection.⁴ Among these, mutations resulting in a hepatitis B e antigen (HBeAg) negative phenotype because of interference with the expression of HBeAg at either the translational level, as in precore stop codon mutants,⁵⁻¹² or at the transcriptional level, as in core promoter mutants,^{13,14} have been associated with fulminant and severe acute hepatitis as well as with recurrent HBV infection after liver transplantation^{15,16} or fatal HBV reactivation following cytotoxic treatment.¹⁷ However, other studies suggest that fulminant hepatitis B is not caused by a specific genomic mutation¹⁸ and show that the mutations described above are also found in patients with acute self-limited or chronic hepatitis B as well as in asymptomatic HBV infection.^{12,22-25}

In this study, we identified and characterized a novel HBV mutant in a heart transplantation recipient who died from fulminant hepatitis B transmitted by the donor.

PATIENTS AND METHODS

Case Report. The patient was a 56-year-old white woman who underwent orthotopic heart transplantation for dilated cardiomyopathy. The donor was a male of Asian origin. After transplantation, donor serum was shown to be HBeAg positive. The recipient was serologically negative for all HBV markers at the time of transplantation; no passive or active HBV vaccine was given. Postoperatively, the clinical course was uneventful under standard immunosuppressive therapy with cyclosporine (200 mg/d), azathioprine (75 mg/d), and prednisone (35 mg/d). In January 1993, 2 months after transplantation, the recipient was serologically HBeAg and HBcAg positive without signs or symptoms of liver disease. In May 1993, 6 months after transplantation, antibodies against hepatitis B core antigen (HBcAg) of the immunoglobulin M type (anti-HBc IgM) were detectable. Antibodies against the hepatitis delta antigen or hepatitis C virus were repeatedly negative. The recipient was still asymptomatic. However, in July 1993, 8 months after transplantation, serum aminotransferase levels were elevated and the recipient became jaundiced. Liver biopsy revealed steatoviral hepatitis. In August 1993, 2 weeks later, the patient died from fulminant hepatitis B. Sera obtained from the donor at the time of transplantation and from the recipient before transplantation and during follow-up were stored at -80°C. Liver tissues obtained from the donor and the recipient at the time of death were formalin-fixed and paraffin-embedded.

Isolation and Cloning of HBV DNA From Serum. HBV DNA was extracted from serum as previously described.²⁶ DNA was isolated from 14 mL serum obtained from the recipient several hours before death. Serum was incubated for 5 hours at room temperature in the presence of 10 mmol/L MgCl₂ and 20 µg/mL of DNaseI (Boehringer Mannheim, Mannheim, Germany) to digest free DNA. After pelleting

Abbreviations: HBV, hepatitis B virus; HBeAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; EDTA, ethylenediaminetetraacetic; HNF-1, hepatocyte nuclear factor 1.

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through a 30% sucrose cushion at 178,000 × g overnight at 4°C, proteinase K digestion in the presence of 1% of sodium dodecyl sulfate (SDS) and phenol/chloroform extractions, the single-stranded region of HBV DNA was repaired by avian myeloblastoma virus reverse transcriptase (Boehringer Mannheim). The repaired double-stranded viral DNA was either digested with *EcoRI* or with *XbaI*, ligated into the appropriately cut plasmid pGEM-7Z(f)+, and cloned in DH5 α cells (BRL, Gaithersburg, MD). For the *EcoRI* and *XbaI* cloning strategies 29 and 30 clones were obtained, respectively, and characterized by restriction enzyme analyses (e.g., *EcoRI*, *XbaI*, *NcoI*). These analyses yielded six different clones: a major clone pF-1 that made up 88% of the clones analyzed. The five minor clones represented 2% each (pF-2, pF-3, pF-4, pF-5) and 4% (pF-6) of the clones, respectively. For transfection experiments, head-to-tail dimers of five *EcoRI*-clones (pF-1 to pF-5) and of one *XbaI*-clone (pF-6) were constructed as described previously.²⁰ The control plasmid padw2 contained a head-to-tail dimer of the wild-type HBV genome subtype adw2 (Okamoto et al. type A²¹), inserted at the *EcoRI* site of pGEM-7Z(f)+.²² A polymerase chain reaction (PCR)-based cloning procedure (see below) was used to generate an additional pF-1 clone without the 11 bp insertion in the core promoter region (pF-1-minus). For the construction of pF-1-minus PCR amplification was performed with *AurII* and *BstEII* 5'-CCGCCTAGGAGGCTCTAGGCATAAAATTCGTGT-3', nt 1775-1802, and *BstEII*-reverse primer 5'-GCTGTAGCTCTTGTCCCAAGAA-TAT-3', nt 2830-2555, followed by digestion with *AurII* and *BstEII*, respectively. The PCR-fragment was introduced into pF-1, using the 4646-nt *BstEII*-to-*NcoI* fragment and the 400-nt *NcoI*-to-*SpeI* fragment, respectively. The removal of the core promoter mutation was verified by DNA sequencing (see below). A replication-competent pF-1-minus dimer was constructed as described above.

PCR Amplification of HBV DNA and Cloning of PCR Products. Amplification of HBV DNA by PCR was performed in the presence of 3 mmol/L MgCl₂, 0.2 mmol/L of each deoxyribonucleoside triphosphate, 25 pmole of each primer (primer sequences can be obtained from the authors upon request), and 1.25 U AmpliTaq (Perkin Elmer Cetus, Norwalk, CT). Amplification was performed with the following three-step cycling profile after an initial denaturation step for 5 minutes at 94°C: 30 seconds denaturing at 94°C, 30 seconds annealing at 55°C, and 60 seconds extension at 75°C for a total of 40 cycles followed by a final incubation for 10 minutes at 74°C. All PCR products were subjected to low melt agarose gel electrophoresis (GIBCO/BRL, Gaithersburg, MD) and visualized by ethidium bromide staining. For cloning, the bands of interest were cut out under ultraviolet illumination, and after short heating to 65°C directly used for cloning (see below). The PCR amplification products were cut with the appropriate restriction enzymes, and cloned into the pGEM-7Z(f)+ (Promega, Madison, WI) or pUC 19 (GIBCO/BRL, Gaithersburg, MD).

DNA Sequencing and Comparative Sequence Analysis. For the six full-length clones pF-1 to pF-6, both strands were sequenced independently. Plasmid DNA was prepared by the lysozyme lysis method and sequenced with HBV specific synthetic primers (Microsynth, Balgach, Switzerland) according to the manufacturer's instructions (T7 Sequencing kit, Pharmacia, Uppsala, Sweden), using ³²S-dATP. In addition, for pGEM-7Z(f)+ clones and pUC 19 clones, SP6, T7, and T7/T3 primers were used. Nucleotide sequences were numbered and aligned at the putative *EcoRI* site as position 1 and compared with 57 complete HBV sequences from the European Molecular Biology Laboratory (Heidelberg, Germany) databank. Sequence analysis was facilitated by the Heidelberg Unix Sequence Analysis Resources (HUSAR) at the German Cancer Research Center, Heidelberg, Germany with standard parameters. The nucleotide sequence data of the six full-length HBV genomes pF-1 to pF-6 reported in this report have been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession nos. X98072 to X98077.

Transfection of HuH-7 Cells and Analysis of HBV DNA. HuH-7 human hepatocarcinoma cells were grown to near confluency at 37°C with 5% CO₂ in Iscove's modified Dulbecco's Medium (GIBCO/BRL), supplemented with 2 mmol/L l-glutamine, penicillin-streptomycin (0.1 million U-0.1 g/L, GIBCO/BRL), and 10% fetal calf serum. Cells were transfected by calcium phosphate coprecipitation of 10 μ g HBV DNA construct, 5 μ g herring sperm DNA, and 2.5 μ g reporter plasmid pCMV/SEAP (TROPIX, Bedford, MA) per 10-cm culture dish. The cells were incubated for 14 hours with the precipitate, washed with phosphate buffered saline, and incubated for another 2 to 5 days. To determine the variability in transfection efficiency, each construct was tested in triplicate. Transfection efficiency was determined 3 days after transfection by a standard colorimetric assay for placental

TABLE 1. Oligonucleotides Used for Gel Retardation Assays

ULC	5'-TTAGTGTGCTTAATAAATAACAATTA-3'
PE56	5'-TCTGCTTAATCATGACATTA-3'
P-1	5'-TCTTTCTACTACTTAATCATAGGACGCTGTA-3'
P-1-mut	5'-TCTTTCTACTACTTAATCATAGGACGCTGTA-3'
P-1-minus	5'-TAGGTTAAAGGCTTTTGTACTAGGACGCTGTAAGGCATAAAT-3'
Pre-S1	5'-CAGAGTATOTAGTAAATCATTAATCCAGAC-3'

Abbreviations: ULC, consensus HNF-1 binding site (underlined); PE56, high affinity HNF-1 binding site from the rat albumin promoter; P-1, putative HNF-1 binding site (underlined) found in pF-1; P-1-mut, pF-1 derived HNF-1 binding site with double mutation (bold); P-1-minus, hphdwt2 core promoter sequence; pre-S1, HNF-1 binding site derived from HBV pre-S1 promoter.

alkaline phosphatase secreted into the medium.²³ For the detection of HBV DNA, viral core particles were isolated 5 days after transfection. Media and cells from two 10-cm dishes were pooled. Cells were lysed by incubation for 15 minutes at room temperature in 400 μ L 1% Nonidet P-40, 50 mmol/L Tris/HCl, 1 mmol/L ethylenediaminetetraacetate (EDTA) (pH 8.0). The cell nuclei were pelleted by centrifugation in an Eppendorf tube at 11,000 rpm for 12 minutes. To digest cloned input DNA, the supernatant was adjusted to 10 mmol/L each of MgCl₂ and CaCl₂ and incubated for 1 hour at 37°C with 20 μ g/mL of DNaseI (Boehringer Mannheim) and 35 U/mL of micrococcal nuclease (Pharmacia, Uppsala, Sweden). Nuclease digestion was stopped by the addition of EDTA (final concentration 30 mmol/L). Viral core particles were digested for 1 hour at 37°C with 1 mg/mL proteinase K in the presence of 1% SDS. Following one extraction with phenol/chloroform and precipitation with ethanol, methylated input plasmid DNA was once more digested for 1 hour at 37°C with 20 U/mL *DpnI* (Boehringer Mannheim). Core particle associated DNA was extracted with phenol/chloroform, precipitated with ethanol and dissolved in 15 μ L 1 mmol/L Tris/HCl, 0.1 mmol/L EDTA (pH 8.3). Nucleic acids were separated on a 1.2% agarose gel without ethidium bromide and vacuum blotted onto Nytran membranes (NY 13 N, Schleicher & Schuell, Keene, NH) for 2 hours. After ultraviolet cross-linking, the blots were prehybridized for at least 4 hours at 42°C in 50% (vol/vol) formamide, 2.5× Denhardt's solution (1× Denhardt's solution: 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin), 5× saline sodium phosphate EDTA (pH 7.4) (1× SSPE: 150 mmol/L NaCl, 10 mmol/L NaH₂PO₄, 1 mmol/L EDTA), 0.1% SDS, and 100 μ g/mL freshly denatured herring sperm DNA. The membranes were hybridized with recombinant full-length HBV DNA, ³²P-labeled by random primed labeling (Boehringer Mannheim) in the above described buffer for 18 hours at 42°C. The blots were washed in 1× saline sodium citrate, 0.5% SDS for 15 minutes at 42°C, in 1× saline sodium citrate, 0.2% SDS for 30 minutes at 58°C, and in 0.1× saline sodium citrate, 0.2% SDS for 20 minutes at 58°C, followed by autoradiography at -80°C.

Gel Retardation Assays. Gel retardation assays were performed as previously described²⁴ with rat liver nuclear extracts or with bacterially expressed 6His-HNF protein, containing the N-terminal DNA binding domain (aa 1 to 286) of rat hepatocyte nuclear factor-1 (HNF-1).²⁴ Double-stranded oligonucleotides used for band-shift experiments (Table 1) were radioactively labelled. Double stranded unlabelled competitor oligonucleotides (Table 1) were added to the reactions at the indicated molar excess. For supershift experiments, two rabbit polyclonal antibodies against HNF-1 were used: α H-193 that is directed against a C-terminal peptide of HNF-1²⁵, and α HNF-283 that is directed against the N-terminal half of HNF-1.²⁶

Serological and Immunohistochemical Analyses. Markers of HBV or hepatitis delta virus infection were detected with enzyme-linked immunosays from Abbott Laboratories, Chicago, IL. Anti-hepatitis C virus was determined with the enzyme-linked immunoassay-2 from Ortho Diagnostic Systems, Tokyo, Japan. Liver tissues from the donor and the recipient, obtained at autopsy, were fixed in 4% phosphate buffered formalin and embedded in paraffin. Immunohistochemical analyses for HBsAg and HBeAg were performed with the respective polyclonal antibodies using the DAKO Pap kit (DAKO A/S, Glostrup, Denmark). Sections were counterstained with hematoxylin.

RESULTS

Genetic Heterogeneity of Serum HBV DNA. We isolated HBV DNA from serum of a heart transplant recipient who died after 9 months from transplant-transmitted fulminant

TABLE 2. Genetic Characteristics of the Major Six Clones From the Heart Transplantation Recipient

Clone	Core Promoter	DR*		Pre-S1 Region		Other Characteristics
	11-bp Insertion	1	2	18-bp Deletion	108-bp Deletion	
pF-1	-	+	-	+	+	see Fig. 2
pF-2	-	-	-	+	+	No X start codon
pF-3	-	-	- [†]	-	-	X ORF (nts 1627-1602): 76 bp deleted
pF-4	+	-	+	+	-	X ORF (nts 1801-1996): 198 bp inserted [‡]
pF-5	+	-	- [†]	+	-	X ORF (nts 1265-1602): 338 bp deleted [§]
pF-6	-	+	-	-	-	Wild-type sequence, donor-like

* DR1 = direct repeat 1; DR2 = direct repeat 2.

† Deletions end just 3' of DR2.

‡ Nucleotides inserted (nt 1603-1800) start just 3' of DR2.

§ Deletion extends 111 bp upstream of the X start codon.

hepatitis B. Conventional cloning and restriction enzyme analysis of 59 clones obtained by two different cloning strategies (*EcoRI* and *XbaI*) revealed six different HBV DNA genomes pF-1 to pF-6 (Table 2 and EMBL Data library, Accession nos. X98072 to X98077). The major HBV DNA species pF-1 represented 88% of the viral population in the patient's serum at the time of death. The five minor clones represented 2% each (pF-2, pF-3, pF-4, pF-5) and 4% (pF-6) of the clones, respectively. The five clones pF-1 to pF-5 contained an 11 bp insertion in the core promoter region as well as an 18 bp and an 108 bp in-frame deletion in the pre-S1 region. No precore stop codon mutation was found in any of the clones. In addition to the 11 bp insertion and the pre-S1 deletions, the five clones pF-1 to pF-5 showed a number of mutations. Clone pF-2, for example, had no X gene start codon because of an ATG to GTC mutation, similar to the X gene start codon mutation (ATG to ATA) previously described.³⁷ Clones pF-3, pF-4, and pF-5 represented defective viral genomes, based on a deletion of the direct repeat DR2 (pF-3 and pF-5) or a duplication in the X open reading frame (pF-4), similar to variants described in patients with posttransfusion hepatitis³⁸ and in woodchucks.³⁹ Different from clones pF-1 to pF-5, clone pF-6 was shown to be a full-length HBV genome without insertion or deletion. As determined by sequence analysis of PCR amplification products, clone pF-6 was similar to HBV DNA found in serum from the donor as well as in sera from the recipient obtained 6 or 3 months before death (data not shown).

Replication Competence of the Clones pF-1 to pF-6. The biological properties of the six mutant viral genomes cloned from serum of the transplant recipient were characterized by transfection of HuH-7 cells with head-to-tail dimers of the clones pF-1 to pF-6. Five days after transfection, DNA was isolated from the cell cultures and analyzed by Southern blot hybridization. As shown in Fig. 1, the two constructs pF-1 and pF-2 showed a higher level of replicative HBV DNA intermediates than the wild-type clone. Densitometric tracing revealed that the level of replication of pF-1 and pF-2 is at least three times higher than that of wild-type padw2. As predicted from the mutations detailed above (Table 2), clones pF-3 and pF-5 with a deletion of the direct repeat DR2 were replication defective. Also, clone pF-4 with a duplication in the X/core promoter region resulting in two DR2 motifs was replication defective *in vitro*. As expected, clone pF-6 showed wild-type levels of viral replication (data not shown).

Nucleotide Sequence and Genetic Organization of the Major HBV Variant pF-1

The complete nucleotide sequence as well as the genetic organization of the predominant viral genome pF-1 obtained from the serum of the transplant recipient shortly before death was analyzed in detail.

Comparison of HBV Genomes. The complete nucleotide sequence of the predominant genome pF-1 is given in Fig. 2. It has a genomic length of 3,100 bp and is of HBV genotype B according to Okamoto et al.³¹ Based on a comparison with 57 complete human HBV sequences deposited in the EMBL data library, the sequence of pF-1 is closest to HBV strain hpbaw2 described by Okamoto et al.³¹ As compared with this strain, a total of 51 nucleotide substitutions were detected. They are distributed throughout the genome. Seven mutations, underlined in Fig. 2, are unique and not previously described in other HBV strains. The 34 nucleotide substitutions result in 39 amino acid substitutions, identified in all four open-reading frames: 6 in the core region, 4 in the X region, 21 in the polymerase region, and 8 in pre-S1/pre-S2/S region.

The pre-S1 and pre-S2 Gene Products. The first of the two start codons of the pre-S1 region at position 2861 was absent in pF-1, because of an 18-bp deletion spanning nucleotides 2863 to 2880. Similar small deletions around the first AUG codon of the pre-S1 region were previously observed in a HBV isolate from a chimpanzee⁴⁰ as well as in human HBV strains of a different genotype, i.e., in all members of genotype D,⁴¹ in three members of genotype C,⁴² and in one member of genotype A.⁴³ In addition, the pre-S1 region showed an 108-bp in-frame deletion spanning nucleotides 2974-3071. The gene product was truncated two amino acids downstream from this deletion because of a TGG to TAG nonsense mutation, similar

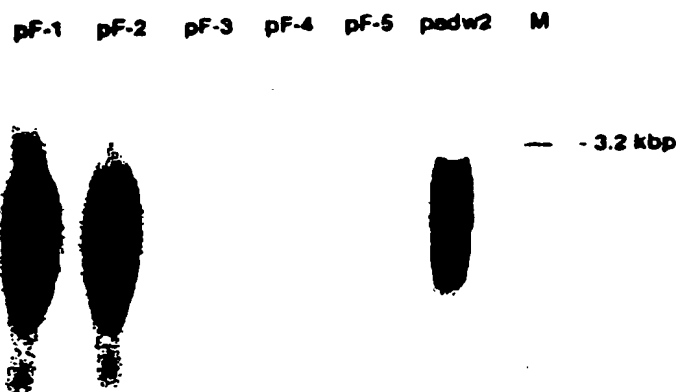


FIG. 1. Replication competence of HBV variants associated with fulminant hepatitis B. Southern blot analysis of cytoplasmic DNA isolated from HuH-7 cells 5 days after transfection with the cloned HBV variant DNAs indicated on top. Plasmid padw2 is used as a control; M, linear 3.2 kbp HBV DNA marker (35 pg). Autoradiography overnight at -80°C.

ATCCAGCACTTTCCAGAAATCTTTCAAGATCCCTGAGTCAGAGCCCTGTACCTTCTGCTGCTGCTTCCAGTTCAGGAACAGTCAAGCCCTGCTCAGAAAT	100
ACTGCTCTCTCCATATCTCTCAATCTTATCCGAAGATTCGGAGCCCTGTCTCTCAACATCGAGACATCTCCATCAGGACTCTCCAGGACCCCTGCTCTGCTTAC	200
AGCTCGGCTTTCTCTCTGACAAAAATCTCTCAAAATACCAATACAGAGCTCTAGACTCTCTGCTGACTCTCTCAATTTCTAGCGGAAACACCCCTGCTGCT	300
TGCGCAAAATTTCTCACTCTCAAAATCTCTCACTCACTCACTCACTCACTCACTCACTCACTCACTCACTCACTCACTCACTCACTCACTCACTCACTCACT	400
TTCTCTGCT	500
CCAGCACTCTGACCATCTCAAACTCTCACT	600
TATCT	700
CT	800
TCT	900
TCT	1000
TCT	1100
TCT	1200
TCT	1300
TCT	1400
TCT	1500
TCT	1600
TCT	1700
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TCT	2100
TCT	2200
TCT	2300
TCT	2400
TCT	2500
TCT	2600
TCT	2700
TCT	2800
TCT	2882
TCT	2953
TCT	2974
TCT	3074
TCT	3100

FIG. 2. Nucleotide sequence of the major HBV DNA clone pF-1. Nucleotide changes with respect to hpbudw2 are indicated by white letters on black background. Unique changes are underlined. Deletions are indicated by dots. Start and stop codons for translation as well as important regulatory sites are bold-faced. The 11 base insertion is marked bold between lines.

to that previously observed in a patient with chronic hepatitis B.⁴⁴ In this case, an 117-bp deletion located C-terminally included the initiation codon of the pre-S2 region. In pF-1, the pre-S2 region was fully conserved and showed no unique amino acid substitutions. Recently, deletions and/or nonsense mutations in the pre-S1 region,⁴⁵⁻⁴⁷ in the pre-S2 region⁴⁸⁻⁵⁰ or in both regions⁵¹⁻⁵⁴ have been identified.

The S Gene Product. The S gene of clone pF-1 showed two unique amino acid substitutions: leucine to proline at position 9 and threonine to alanine at position 126. The amino acid substitution at position 126 is located in the common C-terminal of the S gene product. Interestingly, Moriyama et al.⁴⁹ and Yamamoto et al.⁵⁴ showed a threonine to serine or a threonine to asparagine substitution at the same position

in patients with chronic hepatitis B who were positive for antibodies to HBsAg (anti-HBs) without previous HBV vaccination and in a healthy HBV carrier who was positive for HBsAg and anti-HBs. At the same position, Okamoto et al.⁵⁵ showed a threonine to asparagine substitution in an actively and passively immunized baby born to a HBsAg positive carrier mother.

The Pre-core and Core Gene Products. No amino acid substitutions and no nonsense mutations were observed in the precore region. In pF-1 HBsAg is made up of only 181 amino acid residues, whereas in all other HBV genomes sequenced to date, it has a length of 183 or 185 amino acids.

The Polymerase Gene Product. The polymerase of pF-1 has two unique amino acid substitutions: aspartate to glutamate

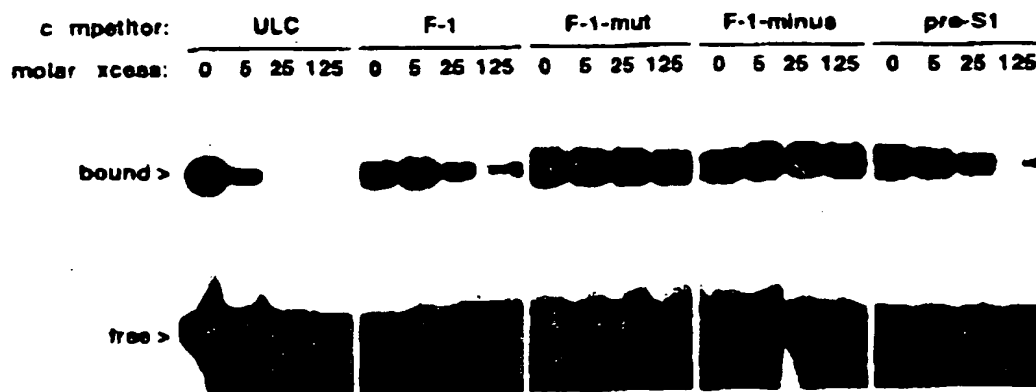


FIG. 3. The 11-bp insertion in pF-1 creates a HNF-1 binding site. Binding of recombinant HNF-1 to a radioactively labeled double-stranded oligonucleotide containing a consensus HNF-1 binding site (ULC) was competed with increasing amounts of the oligonucleotides indicated on top. For nomenclature and sequences see Table 1.

at position 30, and asparagine to aspartate at position 139. Both mutations are located in the terminal protein region. Two in-frame deletions (positions 182-187 and 219-254) were located in the spacer region.

The X Gene Product. The X open reading frame showed a unique alanine to threonine substitution at position 102. In addition, an 11-bp insertion between nucleotides 1777 and 1778 results in a frameshift, creating three novel amino acids and truncating the carboxy-terminus of the X protein by 17 amino acids. The insertion leads to the loss of the cysteine at position 137, which is located in the putative Kunitz domain-like sequence.⁶⁶

Regulatory Elements. No mutations were identified in the sequences relevant for transcription and replication,⁶⁷ except for the HNF-1 binding site in the pre-S1 promoter and the sequence of the LSE/core promoter. Both sequences showed a one nucleotide substitution, underlined in Fig. 2. An 11-bp insertion is located within the basal core promoter.

The 11 Base Pair Insertion in the Basal Core Promoter Generates a New HNF-1 Binding Site

The 11-bp insertion located within the basal core promoter creates together with the flanking sequences (Fig. 2) a putative HNF-1 binding site.³³ To test whether this 11-bp insertion generates a binding site for HNF-1, two sets of experiments were performed. First, binding of recombinant HNF-1 to a labeled double-stranded oligonucleotide, containing the consensus HNF-1 binding site ULC, was competed by different unlabeled oligonucleotides. Binding of HNF-1 to the probe was inhibited by an excess of unlabeled F-1 oligonucleotide (Fig. 3 and Table 1). The oligonucleotide F-1 mut, carrying a double mutation in the 11-bp insertion, as well as oligonucleotide F-1-minus without the 11-bp insertion did not significantly interfere with the binding of HNF-1 to the probe (Fig. 3 and Table 1). The affinity of the oligonucleotide F-1 for recombinant HNF-1 was comparable with that of the well established HNF-1 binding site in the pre-S1 promoter (adw2 pre-S1 in Fig. 3). Second, we tested whether a rat liver nuclear extract can shift oligonucleotide F-1. The assay was validated by a shift with rat liver nuclear extract induced by the proximal element (PE56; Table 1) of the rat albumin promoter, representing a high-affinity HNF-1 binding site (Fig. 4, PE56, lane 1). Labeled oligonucleotide F-1 yielded a complex in the expected position (Fig. 4, F-1, lane 1). In addition, oligonucleotide F-1 mut formed a similar complex, albeit at reduced affinity (Fig. 4, F-1-mut, lane 1), whereas oligonucleotide F-1-minus could not be shifted (Fig. 4, F-1-minus, lane 1). To confirm the specificity of the band-shifts, the complexes formed were supershifted with the polyclonal HNF-1-specific antibody arH-183 (Fig. 4, lanes 2, 5, and 8). The polyclonal HNF-1-specific antibody aHNF-283

abolished the band corresponding to the HNF-1/DNA complex (Fig. 4, lanes 3, 6, and 9). In all supershift experiments, both polyclonal sera lead to an additional nonspecific band (Fig. 4).

Enhanced pF-1 Replication Depends on the New HNF-1 Binding Site

To test whether the new HNF-1 binding site in the basal core promoter of clone pF-1 affects the level of viral replication, a clone without the 11-bp insertion was constructed (pF-1-minus). As shown in Fig. 5, pF-1-minus, indeed, shows only wild-type levels of viral replication.

Accumulation of Mutations during Fulminant Hepatitis B

The HBV core upstream regulatory sequence, the core promoter, and the pre-core region were PCR-amplified with

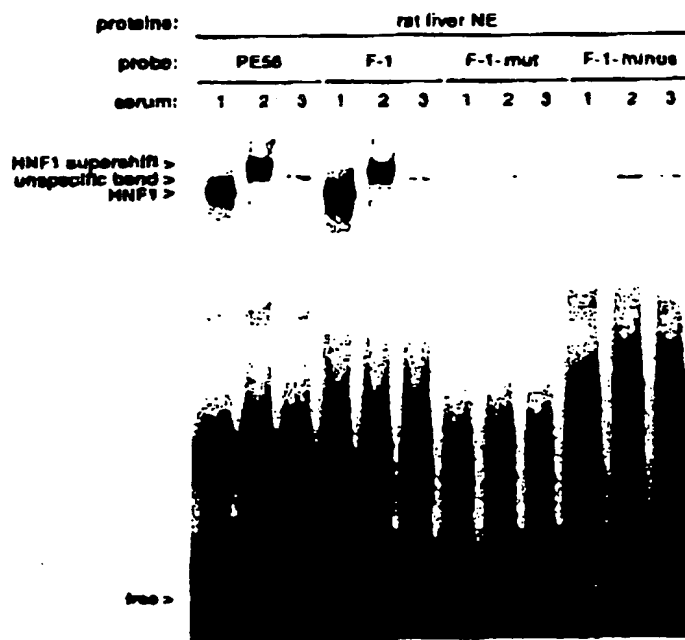


FIG. 4. The pF-1 specific core promoter sequence binds to HNF-1 present in rat liver nuclear extracts. Radioactively labeled double-stranded oligonucleotides (probe) were shifted with nuclear extracts (rat liver NE) in the presence of fetal calf serum (1) and supershifted with anti-HNF-1 rabbit polyclonal antibody arH-183 (2) or aHNF-283 (3). For probe nomenclature and sequences see Table 1.

M pF-1-minus pF-1 padw2

3.2 kbp -



FIG. 5 Effect of novel HNF-1 binding site on HBV replication. Southern blot analysis of cytoplasmic DNA isolated from HuH-7 cells five days after transfection with the cloned HBV variant DNAs indicated on top. Plasmid padw2 is used as a control; M, linear 3.2 kbp HBV DNA marker (35 pg). Autoradiography overnight at -90°C .

primer pair 1/2 in serum samples obtained from the donor and from the recipient at three different time points after transplantation. Twenty-three clones were sequenced: 6 clones from a serum sample of the donor, 7 clones from a serum sample of the recipient taken in February 1993, 6 clones from a serum sample of the recipient taken in May 1993, and 4 clones from a serum sample of the recipient taken shortly before death, in August 1993.

The nucleotide sequences of the core upstream regulatory region and the core promoter are given in Table 3. The nucleotide sequences were identical in all clones obtained from the HBV-infected donor and from serum samples of the recipient taken in February and May 1993, respectively. The sequences of the four clones obtained from the patient in August 1993 were identical to the sequence of the major clone pF-1 and differed at five positions from the earlier serum samples of the recipient and of the donor. The 11-bp insertion creating the novel HNF-1 binding site in the core promoter region could be identified only in August 1993 at the time of fulminant hepatitis B. All four clones from this latest serum sample displayed hpbadw2 wild-type sequences at positions

1762_A, 1764_C, and 1899_G, respectively, whereas all other clones showed nucleotide substitutions compared with hpbadw2 at the respective positions. None of the 23 clones showed a pre-core nonsense mutation.

In the PCR products obtained with primer pair 1/2, mutations were also identified in the core gene (Table 4). Donor and early samples from the recipient displayed highly homologous core sequences. Again, the latest serum taken at the time of fulminant hepatitis B differed considerably from all previous samples. Most of the mutations were located in the sequences encoding the N-terminal part of the core protein and were clustered in the major T-helper cell epitope.⁵⁶

Immunohistochemical Analysis of Liver Tissue

Immunohistochemical analysis of the donor liver showed diffuse and abundant expression of HBsAg in the cytoplasm of the hepatocytes (Fig. 6A). By comparison, at the time of fulminant hepatitis HBsAg expression was very scattered in the recipient (Fig. 6B). HBcAg expression was focal and nuclear in the donor (Fig. 6C). By contrast, in the recipient very strong nuclear as well as cytoplasmic HBcAg staining was identified (Fig. 6D).

DISCUSSION

In the present investigation, we characterized 59 HBV clones obtained by conventional cloning from serum of a heart transplant recipient who died after 9 months from transplant-transmitted fulminant hepatitis B. By restriction enzyme analyses, the clones obtained could be categorized into six different genomes: pF-1, representing approximately 90% of the clones, and pF-2 to pF-6, each representing approximately 2-4% of the viral species. Clone pF-6 was similar to HBV DNA found in serum of the infectious donor and corresponded to hpbadw2 wild-type HBV DNA. By comparison, the clones pF-1 to pF-5 carried an 11-bp insertion in the basal core promoter region of HBV as well as an 18-bp and a 108-bp insertion in the pre-S1 gene.

To identify HBV DNA sequences responsible for the fulminant clinical course in our patient, replication-competent constructs of the six viral genomes were constructed and transfected into HuH-7 cells in parallel with the HBV wild-type construct padw2. In cells transfected with the pF-1 and pF-2 elevated levels of viral replication were observed as compared with cells transfected with padw2. No viral DNA was present in cells transfected with pF-3, pF-4, and pF-5 that carried X region deletions or duplications. Apart from the pre-S1 deletions, the major mutation common to pF-1 and pF-2 was an 11-bp insertion in the core promoter region, suggesting that it could be the basis for enhanced viral replication. Indeed, removal of the 11-bp insertion from pF-1 (pF-1-minus) lead to a reduction in viral replication to HBV DNA wild-type levels.

Based on gel retardation experiments, the 11-bp insertion in the core promoter region was shown to generate a novel

TABLE 3. Nucleotide Changes in the Core Upstream Regulatory Sequences

	1632	1661	1677	1726	1763	1775	1899
hpbadw2	CGG	GGA	AGC	AAT	TTAAAGGTCCTTCTACTAGGAGG		GTC
D1-D6	A	A		C	T.A.		C
Feb F1-F7	A	A		C	T.A.		C
May F1-F6	A	A		C	T.A.		C
Aug F1-F4	A	A	A	C	GTTAATCATTA		

NOTE. PCR amplified fragments from serum samples of the transplant donor (D) and the recipient (F) at different time points after heart transplantation (February, May and August 1993). Positions of the respective nucleotides in the reference HBV hpbadw2 are given at the top. Data indicate identity to hpbadw2. The 11 bp insertion in the August samples F1-F4 is shown at the bottom.

TABLE 4. Nucleotide Substitutions in the Core Gene

hpbcdw2	1913	1975	1979	2003	2012	2063	2147	2188	2237	2351	2363	2492
	CCG	TCC	GTC	TCT	TAT	CTC	GAA	ATC	GAA	CGA	TCC	CAA
D1-D6	...	G..t	...t	A..	..C	T...	C..	..A.	A..	...
Feb F1-F7	...	G..t	...t	A..	..C	T...	C..	..A.	A..	...
May F1-F6	...	G..t	...t	A..	..C	T...	C..	..A.	A..	...
Aug F1-F4	A..	...t	A..t	A..	C..	T..

NOTE: PCR amplified fragments from serum samples of the transplant donor (D) and the recipient (F) at different time points after heart transplantation (February, May, and August 1993). Positions of the respective coding triplets in the reference HBV hpbcdw2 are given at the top. Dots indicate identity to hpbcdw2. Capital letters and lower case indicate missense and silent substitutions, respectively.

HNF-1 binding site. HNF-1 binding sites are typically found in promoters leading to liver-specific gene expression as well as in promoter and enhancer regions of hepatotropic viruses such as in the pre-S1 promoter of HBV⁶⁰ and in the duck hepatitis B virus enhancer.⁶¹ Recently, Fourel et al.⁶¹ (personal communication, May 1996) could show that in woodchuck hepatitis virus infection the amount of pregenomic RNA and the level of viral replication are controlled through a HNF-1 binding site located within the core promoter upstream regulatory sequence.

Recently, additional putative HNF-1 binding sites in the basal core promoter generated by nucleotide insertions have been found in HBV genomes isolated from a chronic HBV

carrier,⁶² from a patient with recurrent HBV infection after liver transplantation⁶³ as well as from immunosuppressed HBV-infected kidney transplantation patients and HBV-infected patients with severe liver disease.⁶⁴ In the chronic HBV carrier, the putative HNF-1 binding site contained a one base substitution compared with our mutant that possibly reduces its HNF-1 binding capacity and therefore viral replication. However, it remains speculative whether sequence variations in the putative HNF-1 binding sites are responsible for the chronic or fulminant course of HBV infection.

In our patient, enhanced viral replication was associated with accumulation of massive amounts of cytoplasmic and nuclear HBcAg in infected hepatocytes, possibly resulting in

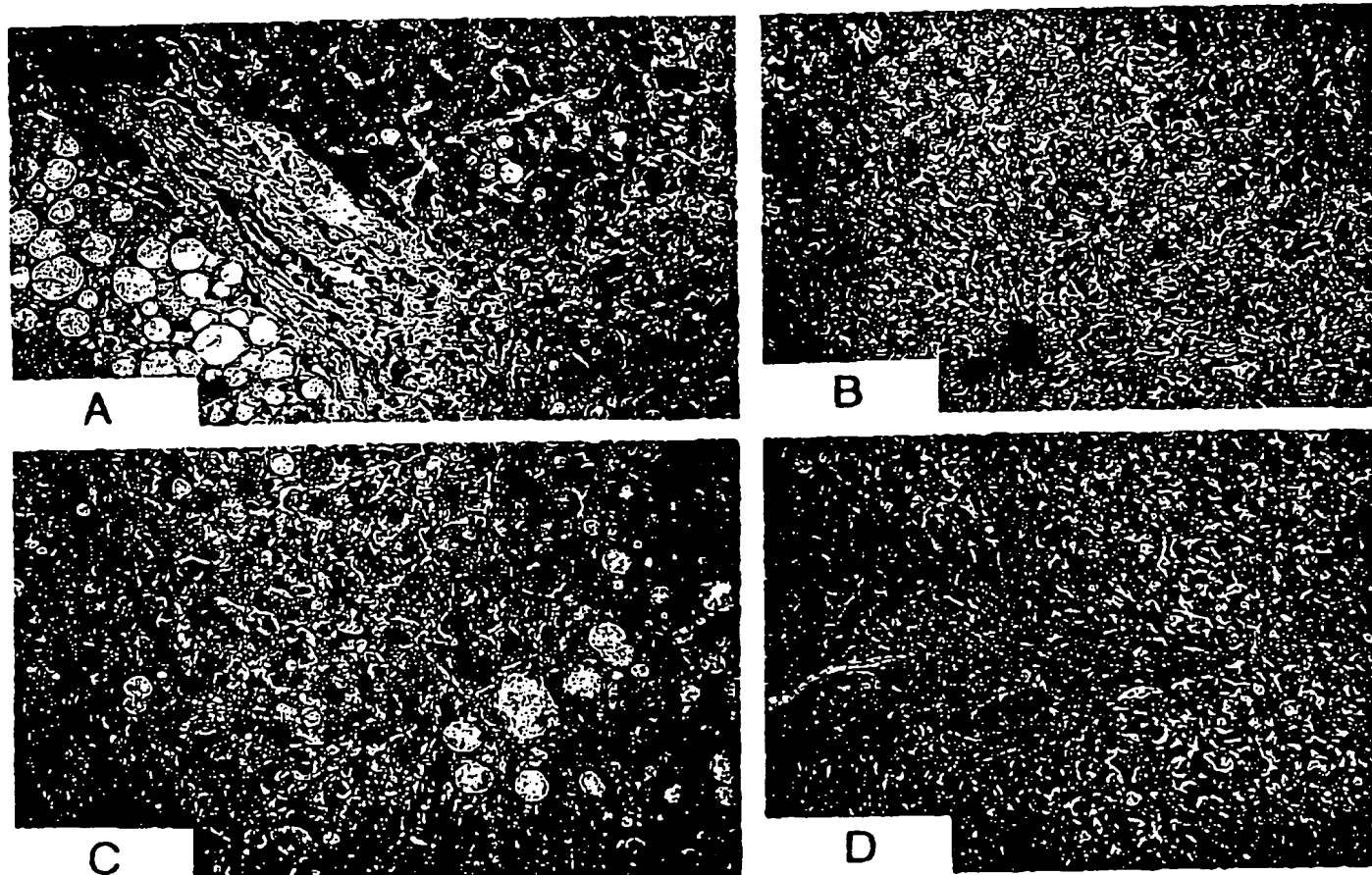


FIG. 6. Immunohistochemical detection of HBsAg and HBcAg in liver from the heart transplant donor (TD) and the patient (F) at autopsy. (A) HBsAg in TD, showing diffuse and abundant cytoplasmic staining. (B) HBsAg in F, showing only few positive hepatocytes. (C) HBcAg in TD, showing focal, nuclear expression. (D) HBcAg in F, showing strong nuclear as well as cytoplasmic staining. Photomicrographs were taken at 160 \times .

cytopathic effects. In a transgenic mouse model it has been shown that overproduction of the large surface antigen is cytopathic (for review see Chisari⁴⁴ and references therein). It is still unclear, however, whether HBV replication or gene expression in infected hepatocytes is directly cytopathic. Again in the transgenic mouse model, high-level HBV replication and HBsAg expression are not cytopathic.⁴⁵ In our patient, in contrast to the hepatic lesions described in patients with fibrosing cholestatic hepatitis and steatoviral hepatitis (see above), immunohistochemically there was almost no accumulation of HBsAg in the cytoplasm of hepatocytes. This may be caused by the one nucleotide substitution in the HNF-1 binding site of the pre-S1 promoter and the truncated pre-S1 gene product because of the 18-bp and 108-bp deletion. Further studies are in progress to assess the capacity of the six genomes pF-1 to pF-6 to produce HBsAg and HBeAg.

In interpreting our findings, we cannot rule out the contribution of other mutations detected in the HBV genomes to the pathogenesis of the fulminant clinical course in our patient. The pathogenic potential of HBV variants with missense mutations in the core gene isolated from patients with sporadic fulminant and severe hepatitis B⁴⁶ as well as in liver transplant patients developing fibrosing cholestatic hepatitis⁴⁷ is unknown. In these cases, immune-mediated mechanisms are postulated to contribute to disease pathogenesis.⁴⁸ In our study, we identified the rapid emergence of new viral quasi-species during fulminant hepatitis B. It cannot be ruled out, however, that these viral quasi-species were already present as a minor viral subpopulation in the donor, not detectable by sequencing of the PCR amplification products. Thus, accumulation of variant viral genomes may have occurred as a consequence rather than the cause of fulminant hepatitis.

Despite these interpretational uncertainties, our results clearly show that a novel viral population different from that present in the infectious source rapidly emerged and accumulated in the transplant recipient during fulminant hepatitis. We identified a novel sequence in the basal core promoter of the HBV genome generated by an 11-bp insertion of a binding site for HNF-1. This insertional HNF-1 binding site mutation was present in nearly all new viral quasi-species present in the patient at the time of fulminant hepatitis B, possibly reflecting a highly pathogenic HBV pool directly involved in the fatal outcome of the patient's liver disease via enhanced viral replication and/or gene expression.

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